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Therapeutic and immunhistochemical evaluation of suicide gene transfer in a rat F98 glioblastoma model: Liposomal approach vs. viral delivery

R. Reszka, J. Zhu, L. Zhang, K. v. Eckardstein*, K. Brand**

Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13122 Berlin
*Institute of Neuropathology, Free University of Berlin, Hindenburgdamm 30, 12200 Berlin
**Max Planck Group, Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13122 Berlin

The therapeutic efficiency of liposome-, retrovirus- or adenovirus-mediated herpes simplex thymidine kinase (HSV-tk) gene transfer, followed by ganciclovir (GCV) administration, was studied in the rat brain tumor model F98.
The suitability of mini-pumps for continuous delivery of DNA-liposome complexes in vivo - in comparison to the single injection - wasinvestigated. The brains of the treated rats were immunohistochemically examined.
In these studies, the cationic liposomal complex DMRIE/DOPE, retrovirus-producing cells or adenoviruses containing the HSV-tk gene were used. The viral or non-viral DNA delivery system was stereotactically administered into the growing F98 tumor. Three days after suicide gene transfer, the animals were treated with ganciclovir at a dose of 100 mg/kg twice daily for 14 days. The rats were sacrificed 3weeks post transfection. Cryosections (8 µm) of the brain were processed histologically by H&E and Nissle staining; sections also underwent immunohistochemical staining with antibodies against glial fibrillary acidic protein (GFAP), CD11 (macrophage and microglia), B- and T-lymphocytes, and the main histocompatibility complexes I and II (MHC I and II), using the alkaline phosphatase-anti alkaline phosphatase (APAAP) method.
No complete tumor regression was noted in the group treated with retroviral gene transfer as well as in the group treated with a single injection of DNA-liposome complexes but the treatments showed a therapeutically significant decrease in the tumor volume. Tumor regression was found in experimental groups treated with pump application of liposomal (4/11) and adenoviral (8/9) gene transfer. In the animals treated with retroviruses, adenoviruses or in the 4 tumor-free animals of the liposome group, the macrophage and T-cell infiltration as well as MHC II reaction seemed to play a major role in the process of dissolving the tumor parenchyma. The adenovirus-treated animals showed significant higher toxicity (edema).
Our results suggest that continuously administered cationic liposomes could be an efficient delivery system for the suicide gene transfer into glioma cells in vivo and represent one of the potential methods for brain tumor gene therapy.

Use of a novel retroviral vector derived from Friend Leukemia virus towards transduction of CD34+ Haematopoietic progenitors : development and regulatory implications

Luz Marina Restrepo*, Jan Bayer*, Michel Masset*, Liliane dal Cortivo**, Jean Pierre Marolleau**, Marc Benbunan**, Christian Gisselbrecht ***, Michel Boiron***** , Michel Marty****, Odile Cohen-Haguenauer*-****#

*Laboratoire Transfert Génétique et Oncologie Moléculaire, Institut d'Hématologie, ** Centre de Transfusion sanguine Paris-Est, *** Service de Réanimation Hématologique, **** Departement d'Oncologie Medicale, ***** Institut de Génétique Moléculaire, Hôpital Saint-Louis, 75475 Paris Cedex 10, France

A novel retroviral vector derived from Friend Leukemia virus towards transduction of CD34+ Haematopoietic progenitors

A novel retroviral vector has been designed based on a Friend-MuLV (Fr-MuLV) FB29 strain (FOCH). The latter has been selected according to characteristics of pathogenicity in mice and high infectivity. In order to investigate potential to genetically engineer haematopoietic precursors, CD34+ progenitors were selected from cord blood, bone marrow and peripheral blood mobilized stem cells (patients + solid tumors) and transduced with FOCH-Neo.

High transduction rates were achieved using virus supernatant and minimal doses of haematopoietic growth factors during pretransduction and transduction steps. A PCR assay investigating the presence of both Neomycine encoding and viral vector sequences tested positive in 45 to 90% of CFU-GM generating cells (bone-marrow and peripheral blood derived cells) following transduction. An average of 35% colonies showed resistant to G418. Such levels of transduction proved reproducible only using supernatants harbouring over 10^7 cfu/ml. In those experiments where long-term in vitro cultures could be maintained over 5 weeks (all cord blood and 5 among 23 PBSC), efficient transduction of LTC-IC haemopoietic progenitors was demonstrated on the basis of both resistance to G418 and virus integration. In the latter case the PCR assay tested positive in as much as 35 to 60% of late unselected CFU-colonies.

Loss in the number of CFU-GM generating cells could clearly be identified during transduction process, as well as in control untransduced duplicate samples. Experiments were thus further directed at minimizing progenitor cell loss in performing short duration of infection. Transduction efficiency proved satisfactory following a single 12 h exposure to virus supernatant 37% CFU-GM colonies harbouring resistance to G418, while duplicate samples exposed to 4 consecutive additions of fresh virus supernatant over 48 hours resulted in 52% G418-R CFUs. This setting is likely to fit clinical transposition since : 1°/ it might spare early progenitors and prevent GF-mediated stimulation of eventually contaminating tumour cells ; 2°/ it could significantly decrease the risks for cell-contamination by adventitious agents ; 3°/ and finally, should result in significant reduction of costs. This novel retroviral vector harbours interesting features towards genetic modification of haematopoietic progenitors. Development of such a novel tool requires implementation of appropriate regulation.

Regulation of Gene Transfer and Therapy

Gene Therapy encompasses medical interventions which involve deliberate modification of the genetic material of living cells. Cells may be modified *ex vivo* for subsequent administration or may be altered *in vivo* by gene therapy products given directly to the subject. When the genetic manipulation is performed *ex vivo* on cells that are administered to the patient, this is also a type of somatic cell therapy. The genetic manipulation may be intended to prevent, treat, cure, diagnose, or mitigate disease or injuries in humans.

In practical, safety and regulatory aspects of gene therapy should be envisaged along three lines : 1st/ experimental and preclinical research ; 2nd/ Manufacturing of gene therapy products under GMP and GLP (Good Manufacturing Practice and Good Laboratory Practices including Standard Operating Procedures (SOPs) and Quality/ Safety Controls (QC) and ; 3rd/ Clinical trials and development, under Good Clinical Practice (GCP). None of these steps truly requires additional legal regulation to already existing ones. What more specifically belongs to gene therapy is the level of complexity resulting from the combination of the various critical levels of concerns. Gene therapy does conjugate basic science, such as gene regulation and gene delivery sytems, with manufacture of biotech products and finally, potential evolution inside the body of a patient following therapeutic intervention.

Oncolytic herpes viruses for brain tumor therapy. E. A. Chiocca, M. Chase, K. Ikeda, N. Qureshi. Massachusetts General Hospital, Boston, MA

Herpes simplex virus type 1 (HSV1) can be engineered to selectively replicate in tumor cells by deletions in viral genes needed for nucleic acid metabolism. Mutant HSV have been generated by homologous recombination into the viral ICP6 (or ribonucleotide reductase) locus. The inserted transgene either encodes for the cyclophosphamide-activating rat cytochrome P450 2B1 gene (mutant designated as rRp450) or for the green fluorescent protein (designated as rRGFP). Another mutant was generated by knock-out of both the ICP6 gene and the GFP gene (designated as MGH1). These mutants can produce killing of rat 9L gliosarcoma or human glioma cells (U87, T98G) at relatively low multiplicity of infection (0.1-0.01). However, the single mutant in ICP6 produced more potent oncolysis than the double mutant. Significant potentiation of rRp450's oncolytic effect was achieved in proliferating cells by addition of cyclophosphamide, without apparent inhibition of viral replication. Since rRp450 maintains an intact thymidine kinase gene, viral replication can be inhibited by the addition of ganciclovir. Taken in conjunction, these new viral mutants should allow us to: 1) follow HSV-mediated gene transfer in vivo, 2) potentiate the oncolytic effect of herpes by the addition of CPA without inhibiting viral replication and spread in the tumor, and 3) use ganciclovir to stop viral replication, if necessary.

A TISSUE-SPECIFIC VIRAL VECTOR BASED ON THE B-LYMPHOTROPIC PAPOVAVIRUS

Michael Pawlita, Monika Oppenlaender, Thomas Neumann and Harald zur Hausen, Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany; Fax: (49) 6221 424932; E-mail: M.Pawlita@dkfz-heidelberg.de

The African green monkey B-lymphotropic papovavirus (LPV), a SV40-related primate polyomavirus, in tissue culture displays a highly selective tropism for a subgroup of human B-lymphoma lines. The viral tissue specificity is determined by the interaction of the viral capsid with the cell surface receptor. We explore whether LPV can be used for the development of a highly tissue-specific viral vector. Helper cell lines were established from the permissive human B-lymphoma cell line BJA-B by stable transfection that express large T-antigen -the viral replication protein- either constitutively or regulated by tetracyclin. After transfection into T-antigen-expressing helper cells recombinant LPV vector genomes, in which the region coding for T antigen has been replaced by the reporter gene chloramphenicol acetyl transferase (CAT) genome replication, structural protein expression, capsid assembly and genome packaging take place and infectious but replication-deficient CAT-LPV particles can be isolated. Upon infection into helper cells CAT-LPV replicates to about the same level as wildtype LPV. From one liter of cell culture about 1.6 x 108 infectious units corresponding to about 33 g capsid protein VP1 have been highly purified. The efficiency and targetting specificity of CAT gene transduction by CAT-LPV particles into various lymphoid and hematopoetic cell lines shall be reported.

RETROVIRUS AND ADENOVIRUS MEDIATED INTERFERON GENE TRANSFER INTO HEMATOPOIETIC STEM CELLS (CD34+) OF NORMAL AND CHRONIC MYELOGENOUS LEUKEMIA

N.G. Abraham

Department of Pharmacology/Gene Therapy. New York Medical College, Valhalla, New York 10595, USA

Gene therapy following gene transfer into hematopoietic cells (CD34+) is now being investigated for several genetic disorders. We have applied a similar approach using interferon gene transfer for treatment of chronic myelogenous leukemia (CML). IFN-α produces a hematologic and cytogenetic response in CML with a survival advantage for cytogenetic responders. Therefore, we examined the effect of transient overexpression of IFN-α using the adenovirus gene transfer approach. The ability of the adenovirus (Adv)-IFN-α gene construct to transfect normal and CML stem cells, CD34+, was examined. The peripheral blood mononuclear fraction from patients with CML treated with G-CSF or GM-CSF/G-CSF was enriched in CD34+ cells. Adv-cytomegalovirus (pCMV) promoter driven IFN-α at multiple doses was assessed to transfect highly purified CD34+ cells in the presence of matrix protein and in co-cultures with the stromal adherent cell layer. The use of cytokines enhances Adv-mediated IFN-α gene transfer into stem cells. Southern blot analysis demonstrated that the Adv-pCMV-IFN-α construct and IFN-α were expressed in cultured CD34+. Transient expression of the IFN-α gene did not suppress proliferation of CD34+ progenitors, CFU-Meg, BFU-E or CFU-CM growth. Reverse transcriptase/polymerase chain reaction (RT/PCR) analysis of RNA from CFU-GM progenitor cells demonstrated transient IFN-α mRNA expression in CD34+ cells. Immunoassay of IFN-α shows that IFN-α suggests that selective expression of IFN-α may be beneficial for CML therapy. We also report on the establishment of novel conditions which permit high efficiency of the retrovirus IFN-α gene into CD34+ cells. Stem cells transfected with retrovirus were infused intravenously to irradiated mice and spleen colony forming units were evaluated for IFN-α marked clones by Southern blot analysis. These studies demonstrate that the retrovirus IFN-α gene can be used to transfect CD34+ cells and possibly for gene therapy of CML.

DOMINANT SELECTION OF GENE-MODIFIED HEMATOPOIETIC CELLS

C. Baum, H.-G. Eckert, M. Hildinger, S. Hegewisch-Becker*, B. Fehse*, A. Zander*, and W. Ostertag.

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie and *Universitätskrankenhaus Hamburg-Eppendorf, both Martinistr. 52, D-20251 Hamburg, FRG. Tel (Fax): +49-40-48051-275 (-187), E-mail:cbaum@hpi.uni-hamburg.de

Therapeutic gene transfer into hematopoietic cells is critically dependent on the evolution of methods that allow ex vivo expansion, high-frequency transduction and also dominant selection of gene-modified long-term repopulating cells. Novel retroviral vectors termed FMEV were cloned which mediate greatly improved gene expression in myelo-erythroid progenitor cells[1]. Transfering the selectable marker multidrug resistance 1 (mdr1), FMEV, in contrast to conventional MoMuLV-related vectors, mediate background-free selectability of transduced human hematopoietic progenitor cells in the presence of myeloablative doses of the cytotoxic agent paclitaxel *in vitro* [2]. FMEV:mdr1-vectors also allow dominant selection of hematopoietic progenitor cells when coexpressing a second gene via internal ribosomal entry or splice. This observation has significant consequences for a number of ongoing and planned gene therapy trials, e.g., stem cell protection to reduce the myelotoxic side effects of cancer-chemotherapy, correction of inherited disorders involving hematopoietic cells, and antagonism of HIV-infection. Methodical and ethical implications of *in vivo* selection strategies will be discussed.

1. Baum et al., J. Virol. 69, 7541-7547 (1995)
2. Eckert et al., Blood 88:3407-3415 (1996)

CHARACTERIZATION OF THE MULTIDRUG RESISTANCE (*MDR1*) GENE AND OF TRANSCRIPTIONAL GENE FUSIONS WITH *MDR1* AS DRUG-SELECTABLE MARKERS IN HEMATOPOIETIC CELLS

T. Licht,[1] J.M. Aran,[2] M.M. Gottesman,[3] I. Pastan,[2] and F. Herrmann.[1]
([1]) Medizinische Klinik der Universität Ulm, D-89081 Ulm, Germany, ([2]) Laboratory of Molecular Biology and ([3]) Laboratory of Cell Biology, National Cancer Institute, NIH, Bethesda, Md., USA.

Expression of transgenes in hematopoietic cells has frequently been found to be low or unstable. Thus, somatic gene therapy of disorders of the hematopoietic system is still considered to be elusive. It has been suggested that chemoresistance genes such as *MDR1* may act as selectable markers, facilitating increased gene expression in transduced cells after exposure to drugs. To study this hypothesis, retroviral vectors were instrumental that contained a full-length *MDR1* cDNA, or a transcriptional fusion of *MDR1* and the glucocerebrosidase gene in which translation of the latter gene was accomplished by an internal ribosomal entry-site (IRES). Following prestimulation with growth factors, murine bone marrow cells were transduced in coculture with retrovirus-producing fibroblasts. Cells were then transferred to fresh medium containing anticancer drugs at various concentrations, and grown for 48-96 hours. Cells were analyzed by flow cytometry for expression of the encoded P-glycoprotein, and for chemoresistance by clonogenic assays. When a producer cell clone was used that synthesized viruses at low titers, *MDR1* gene expression was low. However, it was increased in a dose-dependent fashion following exposure to daunomycin or colchicine. Similarly, chemoresistance of progenitors was increased after drug selection. Increased *MDR1* expression was also observed in cells transduced with the bicistronic vector following selection with anticancer drugs. We then investigated whether drug-treated cells were capable of rescueing lethally irradiated recipient mice. Colchicine-selected bone marrow cells engrafted in recipient mice. Transplantation of drug-selected cells resulted in increased P-glycoprotein expression, and chemoresistance in the marrow of recipients. In conclusion, drug selection of *MDR1*-transduced bone marrow cells facilitates increased gene expression and chemoresistance both *in vitro* and in recipient mice following bone marrow transplantation. Transfer of *MDR1* may be helpful for gene therapy to enhance gene expression with the use of fusion vectors in which *MDR1* is combined with non-selectable genes which correct genetically determined diseases. Drug selection of *MDR1*-transduced progenitor cells may also restore gene expression *in vivo* if expression levels decrease. We intend to study in a clinical trial whether *MDR1* gene transfer can protect hematopoietic cells of cancer patients from the toxicity of intensive chemotherapy.

INCREASED TRANSDUCTION EFFICIENCY OF PRIMARY HEMOPOIETIC CELLS BY PHYSICAL CO-LOCALIZATION OF RETROVIRUS AND TARGET CELLS

M Hutchings*, D Dilloo*, T Hoffman* S Kimborough*, MK Brenner, HE Heslop St Jude Children's Research Hospital, and University of Copenhagen.

Efficient gene transfer into hemopoietic stem cells offers a number of therapeutic applications. However the relatively low titre of retroviral supernatants and the requirement for cell division to ensure integration has meant that transduction efficiency has been low. We have modified a "flow through" approach to cell transduction (Human Gene Therapy 7:743, 1996) and have been able to increase gene transfer efficiency into human hemopoietic progenitor cells. We transduced cells with retroviral vectors encoding a truncated nerve growth factor receptor (NGFR) or neo. Retroviral supernatant was pulled through 0.2 æ polycarbonated membranes followed by placement of cells on the filter. In the absence of cytokines, the transduction efficiency of CD34 cells with a NGFR vector was increased 3-11 fold over that obtained at an identical MOI in liquid culture to produce 11-44% transduction. Furthermore both Thy1+ and Thy1- subsets were transduced with similar efficiency. Similarly transduction with a neo vector as measured by G418 resistance in clonogenic assays increased 1.5 to 5 fold. The mechanism by which gene transfer is improved may reflect co-localization of cells and retrovirus. Costaining of cells transduced on the filter with an NGFR retrovirus with both an NGFR antibody and a gp70 antibody that recognizes viral coat protein revealed high level co-expression. These levels of in-vitro gene transfer are equivalent to those observed when CD34 cells are co-cultured with cytokines. Gene marking studies using distinguishable retroviral vectors will provide a means of learning the effects of flow though transduction on the efficiency of gene transfer to human marrow repopulating cells.

Retroviral Vectors for Overexpression of the DNA-Repairprotein O^6-Methylguanin Methyltransferase (MGMT) in Hematopoietic Cells

M. Jansen, H.Steininger, W. Bardenheuer, M. Flasshove, T. Moritz, S. Seeber Internal Clinic (Cancer Research), West German Cancer Center, University of Essen Medical School, Essen, Germany[1]

Overexpression of MGMT in hematopoietic cells has been shown by various groups to protect from nitrosourea induced hematotoxicity *in vitro* and *in vivo*. While most studies so far have focussed on BCNU, BCNU dose escalation within high dose chemotherapy regimen is problematic, mainly due to its pulmonary toxicity. Therefore, we investigated alternative substances such as CCNU and ACNU. Initial *in vitro* studies were performed in primary clonogenic murine bone marrow cells using an N2/Zip based retroviral vector expressing wildtype (wt) MGMT driven by the human phosphoglycerokinase promoter. Protection achieved for CCNU and ACNU was similar to BCNU. While LD50 for BCNU was 45,9 +/- 24,1 µM in MGMT transduced cells versus 14,0 +/- 3,2 µM in mock transduced controlls (average increase 3,1 +/- 1,1), data for CCNU were 2,1 +/- 0,8 µg/ml versus 0,4 +/- 0,3 µg/ml (average increase 6,4 +/- 2,8) and for ACNU 15,2 +/- 0,6 µg/ml versus 2,6 +/- 2,0 µg/ml (average increase 12,3 +/- 9,6; 3 independant experiments for each substance). We currently establish murine models for CCNU- and ACNU-induced hematotoxicity as a prerequisite for *in vivo* studies.

In summary, drug resistance conferred by MGMT overexpression is not restricted to BCNU. This finding may help to design nitrosourea-containing high-dose chemotherapy protocols circumventing the dose-limiting pulmonary toxicity of BCNU.

RETROVIRAL INFECTION ON FIBRONECTIN: PRECLINICAL STUDIES. H Hanenberg*, K Hashino#, H Konishi#, I Kato#, DA Williams*^. *Wells Center, Riley Hospital for Children, Indianapolis, IN; #Takara Shuzo Co. Ltd., Otsu, Japan; ^Howard Hughes Medical Institute, Indianapolis, U.S.A.

We have previously demonstrated that efficient retroviral gene transfer (GT) into hematopoietic stem and progenitor cells can be achieved with retrovirus (RV) containing supernatant (SN) by colocalizing RV and target cells on specific adhesion domains of recombinant fragments of the extracellular matrix molecule fibronectin (FN) (Hanenberg et al. Nat. Med.

2, 1996: 876). We applied this technology to target primary human hematopietic cells. First, we analyzed gene transfer into T cells which constitutively express the FN receptors VLA-4 and VLA-5. Human peripheral blood (PB)-derived mononuclear cells (MNCs) were prestimulated for 2-3 days with CD3 and IL2 and then infected for 2 days with an amphotropic RV containing the murine ADA cDNA on the FN fragment CH-296. CH-296 contains the binding sites for VLA-4 (=CS1) and for VLA-5 (=CBD). After 4 days, cell cultures contained >80% T cells, the majority of which were strongly activated as indicated by their HLA-DR expression. Analysis of GT efficiency by ADA isoenzyme assay showed the activity of the murine ADA protein equal to the endogenous human ADA protein in the unselected population. Next, we analyzed gene transfer into human clonogenic CD34+ cells obtained from bone marrow (BM) or from PB after mobilization with G-CSF. The integrin receptors VLA-4 and VLA-5 are both expressed on hematopoietic precursor cells and are thought to be involved in homing of these cells in the BM microenviroment. CD34+ BM cells were prestimulated overnight with SCF and IL6. The next day, plates were coated with increasing concentrations of CH-296 and cells resuspendend in SN containing an amphotropic NEO RV were added. GT efficiency assessed 12-16 days later as the number of $G418^r$ colonies did not increase significantly with coating concentrations above 8 $\mu g/cm^2$. Increasing the cell numbers from 500 to 625,000 per cm^2 revealed that the GT efficiency was not influenced by the MOI over a range of 3 logs suggesting that the amount of RV particles present is not a limiting factor for transduction of CD34+ cells. We then investigated the role of cytokine prestimulation in the transduction protocol. To this end, CD34+ PB cells were prestimulated with various cytokine combinations for 1 or 2 days or directly put in the presence or absence of cytokines on CH-296 coated plates with NEO34 RV. GT was most efficient when using a 40h prestimulation period followed by two 4 hour transduction periods. The optimal cytokine mixture was the combination of SCF, G-CSF and c-mpl ligand achieving gene delivery of up to 70 % of the clonogenic CD34+ cells. These data demonstrate that genetic transduction of primary cells on CH-296 may be well suited for genetic modifications of hematopoietic cells with retroviral vectors in clinically applicable protocols.

Xenograft models for pediatric malignancies - Where do we stand? *J. Vormoor, G. Baersch and H. Jürgens.* Dep. of Ped. Hematol./Oncol., U of Münster, Germany.

Xenograft models using immune-deficient mice have been established for a variety of pediatric malignancies, particularily leukemias. We have recently been able to improve the ALL xenograft model by using NOD-SCID instead of classical SCID mice as recipients for human leukemic cells (Baersch et al., submitted). As few as 10,000 ALL cells from a patient with pre-pre-B-ALL were sufficient to reproduce the disease in mice.

Interestingly, human tumor cells seem also to be able to grow in immune-deficient mice in a manner similar to that in patients. A scientist from the Childrens Hospital Los Angeles could demonstrate that human neuroblastoma cells after intravenous injection disseminate to the liver, adrenal glands and bone marrow of transplanted SCID mice (Int. J. Cancer 67:379, 1996). Our own preliminary results suggest that Ewing tumor cells after intravenous injection can cause bone tumors in transplanted NOD-SCID mice. This orthotopic growth represents a great improvement to the unphysiolocial subcutanous tumor growth in nude mice.

Strategies will be discussed how these NOD-SCID mice models can now be used to study homing/metastasis of malignant cells and to identify normal and malignant stem populations in pediatric leukemias and solid tumors.

Treatment of Malignant Gliomas - Experience of a Phase I/II Clinical Study

D. Klatzmann - Laboratoire de Biologie et Thérapeutique des Pathologies Immunitaires, CERVI Hopital de la Pitié-Salpetrière, Paris, FRANCE

Glioblastoma might be one of the best tumor to be treated by gene transfer of an HSV1-TK suicide gene and ganciclovir administration. In this setting, the treatment could act as a molecular scalpel to selectively destroy residual tumor cells after surgical debulking. We initiated a phase I/II to clinical trial in patients with glioblastoma using this therapeutic system. Primary objectives of the study were to evaluate tolerance to treatment and efficacy based on tumor evolution analysed by MRI and on the overall survival of patients. Preliminary results of this study indicate that the treatment is very well tolerated, and that there exist radiological signs of an antitumor effect of the treatment.

Prodrug-activating gene therapy. E. A. Chiocca, M. Aghi, M. X. Wei, C. Kramm, X. O. Breakefield (Massachusetts General Hospital, Boston), T. C. Chou (Memorial Sloan Kettering, New York), O. M. Colvin (Duke).

Intratumoral transfer of prodrug-activating genes has been pursued as a strategy for improving the therapeutic index of chemotherapy agents. We first described the use of the rat cytochrome P450 2B1 gene to provide tumor cell sensitivity to cyclophosphamide (CPA) (Wei et al., 1994). This gene can also provide chemosensitivity to a pro-nitrosourea agent (MPCNU). The proliferation of glioma cells that express the transgene is rapidly inhibited by these prodrugs. The activated CPA metabolite is diffusible, thereby providing bystander killing even at a distance. Antitumor effects can be achieved using retrovirus, adenovirus, or herpes mutants modified to express P450 2B1. Using the median-effect method of Chou, synergistic interactions between CPA and ganciclovir were observed in cells co-expressing the P450 and the TK genes. These results provide a framework for exploiting combinations of prodrug-activating genes to generate more potent antitumor effects.

ADENOVIRALLY TRANSFERRED p16*INK4/CDKN2* AND p53 GENES COOPERATE TO INDUCE APOPTOTIC TUMOR CELL DEATH

Volker Sandig[1], Karsten Brand[1], Susanne Herwig[1], Jiri Lukas[2], Jiri Bartek[2] and Michael Strauss[1,2]

[1]Max-Planck-Gesellschaft, Humboldt-Universität, Max -Delbrück-Centrum for Molecular Medicine, Robert-Rössle-Str. 10. D-13122 Berlin-Buch, Germany

[2]Danish Cancer Society, Division for Cancer Biology, Department of Cell Cycle and Cancer, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

Abstract

Repression of cell cycle progression by tumor suppressors might provide a means for tumor therapy. Here we demonstrate that ectopic overexpression of the p16*INK4/CDKN2* tumor suppressor from an adenovirus vector in various cell lines results in block of cell division and, subsequently, in a gradual reduction of pRb levels. Additional overexpression of p53, but not p53 on its own, induces apoptotic cell death. Simultaneous adenoviral transfer of p16 and p53 genes leads to inhibition of tumor growth in nude mice. These results suggest that combined delivery of two cooperating genes like p16 and p53 could be the basis for a successful strategy of tumor gene therapy.

Betulinic acid triggers CD95 (APO-1/Fas) and p53 independent apoptosis: a pathway to overcome drug resistance

Simone Fulda, Marek Los and Klaus-Michael Debatin

Hematology/Oncology, University Children's Hospital and Division of Molecular Oncology, German Cancer Research Center, Im Neuenheimer Feld 150, 69120 Heidelberg, Germany.

Betulinic acid (BA), an extract from white birch trees, has recently been described as a novel cytotoxic agent against melanoma. To investigate whether or not BA also exhibits cytotoxic activity on other tumors we screened a panel of tumor cell lines for sensitivity to BA and analyzed the molecular requirements for BA mediated cell death. BA is a potent inducer of apoptosis in neuroectodermal tumors such as neuroblastoma, medulloblastoma and ewing sarcoma cells, whereas breast carcinoma, colon carcinoma, small cell lung carcinoma, renal cell carcinoma and T-cell leukemia cells do not respond to BA. BA triggers apoptosis pathways different from those that we previously identified for standard cytotoxic drugs since BA induced apoptosis is independent of CD95 ligand/receptor interaction and independent of accumulation of wild-type p53 protein. ICE/Ced-3 like proteases are crucially involved in BA induced apoptosis since inhibition of ICE proteases by the peptide inhibitor zVAD-fmk completely blocks BA triggered apoptosis. In addition, the prototype protease CPP32/YAMA/Apopain and the prototype ICE substrate PARP are proteolytically processed. Bax and Bcl-x_s, two death promoting proteins of the Bcl-2 family, are upregulated following BA treatment changing the ratio of pro-apoptotic to anti-apoptotic Bcl-2 related proteins. Most importantly, neuroblastoma cells resistant to CD95 and doxorubicin respond to treatment with BA suggesting that BA may overcome drug resistance of neuroblastoma. Since BA is also active on neuroblastoma cells *ex vivo*, BA may become a promising new agent for the treatment neuroblastoma *in vivo*.

The HSV-TK/Ganciclovir- and E. coli CD/5-Fluorocytosine-Systems in Experimental Brain Tumor Gene Therapy

CM Kramm[1], Fischer U[1], Genutt P[1], Aghi M[2], Chase M[2], Rainov N[2], Chiocca EA[2], Breakefield XO[2], Burdach St[1].

[1] Laboratory for Experimental Hematology and Stem Cell Transplantation, Department of Pediatric Oncology and Hematology, Heinrich-Heine-University, Düsseldorf, Germany

[2] Molecular Neurogenetics Unit and Molecular Neurooncology, Massachusetts General Hospital and Harvard Medical School, Charlestown/Boston, USA

Combination of chemotherapeutic drugs is one of the main principles for treatment of neoplastic diseases. The concept of poly-chemotherapy is based on the utilization of synergistic drug effects and the prevention of drug resistance. In the present study, we have tried to transfer this concept to pharmacological gene therapy for experimental brain tumors. We have generated a rodent gliosarcoma cell line, 9L-cd/tk, with stable expression of the genes for HSV thymidine kinase (HSV-TK) and E. coli cytosine deaminase (CD). Both genes combined with ganciclovir, respectively 5-fluorocytosine treatment are the most widely used paradigms in pharmacological cancer gene therapy. In cell culture, the combined use of both systems showed a significant synergistic effect. Preliminary experiments in

nude mice suggest that the combination of both drugs may also be superior in vivo to the treatment with each single drug. Based on previous findings that HSV-TK- and CD-mediated pharmacological gene therapy induces apoptosis in tumor cells, we studied in various 9L lines the expression of APO-1/FAS under treatment with ganciclovir or 5-fluorocytosine. The activation of APO-1/FAS by its ligand is the best investigated apoptotic pathway to date. In 9L-cd/tk cells and 9L cells transduced with HSV-TK alone (9L-tk), APO-1/FAS expression was increased by ganciclovir. The same effect was also observed in 9L-cd/tk cells during 5-fluorocytosine treatment. 9L-tk cells showed no APO-1/FAS upregulation by 5-fluorocytosine. Native 9L cells or 9L cells transduced with a non-therapeutic gene showed no APO-1/FAS expression neither with ganciclovir nor fluorocytosine. Interestingly, 9L-tk/cd cells showed a high APO-1/FAS expression without any drug treatment, whereas parental cell lines, native 9L and 9L-tk, did not display such a baseline APO-1/FAS expression. Further experiments will show if this non-induced APO-1/FAS expression renders 9L-cd/tk cells more susceptible to apoptotic stimuli than 9L or 9L-tk cells without APO-1/FAS expression, and if this phenomenon is a transfection and/or selection artefact or represents a molecular correlate for the synergism of both pharmacological gene therapy systems.

Title: **Clinical Experience of Retroviral Mediated HSV-TK-Gene Transfer in Patients with Glioblastoma Multiforme-**

Authors: F. Weber, H. Bojar, H. B. Priesack, F. Floeth, D. Lenartz, J. Kiwit, W. Bock

Address: Neurochirurgische Klinik Heinrich Heine University Düsseldorf Moorenstr. 5, D-40225 Düsseldorf, Germany
Institute of Oncological Chemistry, Heinrich Heine University Düsseldorf, Moorenstr. 5, D-40225 Düsseldorf, Germany

The study GLIB 201-E-00 (Sandoz / GTI) was designed as a prospective, open label, multicenter phase II study. It consists of two treatment strategies:

- Stereotactical injection in primary glioblastomas
- Administration of vector producing cells after open tumor surgery

As objectives primarily the safety of administration of VPC followed by gancyclovir treatment in subjects with recurrent glioblastoma multiforme and secondary an evidence of antitumor efficacy as assed by survival as well as time to progression, tumor response and functional status should be evaluated.

The treatment strategy was scheduled in the folowing way:

After tumor debulking the VPC were injected into the cavity adjacent area, a total volume of 10ml in 50 single injections with a concentration of 1 x 108 cells/ ml was administrated. 14 days after surgery, the the gancyclovir treatment was started for 14 days. The treatment was completed at day 28 after surgery. Main eligible criteria were:

- confirmed GM (new / relapsed)
- age between 18 and 75
- patients with recurrence must have completed any anticancer treatment at least 4 weeks prior to study entry

patients were excluded when:

- the tumor involves the brainstem, both hemisheres or multifocal disease
- the tumor mass exceeds 30 cc in volume
- diffuse subependymal or CSF dissemination
- has a prior history of brachytherapy or gamma knife treatment
- has a history of an active infection or history of HIV
- they suffer from severe systemic disease or prior malignancy
- or women who are pregnant, are lactating , or do not perform a contraception

In our center 10 patients suffering from a recurrent GBM were treated.. The treatment was well toleratedThere were no side effects related to the application of VPC intracerebrally. 5 patients are still alive,one presenting a complete remission one year after treatment, all other patients have progressive disease but still a significant quality of live. Reviewing the follow up MRI scans the tumor growth continued in those areas were resection had been incomplete or injection of VPC was insufficient.

Interleukin-7 deficiency prevents development of non-lymphoid inflammatory bowel disease.

U von Freeden-Jeffry, RJ Wiler, NJ Davidson, S Burdach, R Murray, Heinrich-Heine-University, Düsseldorf; DNAX Research Institute, Palo Alto, CA

Genetic analysis in mice has shown that, among the cytokines that interact with the common g-chain receptor, Interleukin-7 (IL-7) is unique in its capacity to expand lymphocyte populations. However, much less is known about the potential in vivo role of IL-7 on myeloid cells. No obvious abnormality in myelopoietic cells was apparent in IL-7 deficient (-/-) mice.

Lymphopenic IL-7(-/-) mice were intercrossed with lymphoid deficient RAG-2(-/-) mutant mice. Adult RAG-2(-/-) but not doubly mutant IL-7(-/-)/RAG-2(-/-) mice developed a severe colitis and in many cases a rectal prolapse. Routine health screening for pathogens revealed the presence of Helicobacter hepaticus (H. hepaticus) in both colonies of animals. The pathology in the RAG-2(-/-) mice was similar to that reported for naturally occuring H. hepaticus infection. In further experiments the infection was induced by cohousing newborn animals of each genotype and by reexposure of antibiotic cured mice to the natural course of infection. WBC counts, platelets counts and serum Amyloid A were used as indices of inflammatory response. The

RAG-2(-/-) mice showed significantly higher values in all parameters than the IL-7(-/-)/RAG-2(-/-) mice. Histologically RAG-2(-/-) mice showed lesions in the cecum consisting of multifocal MHC class II positive epithelial hyperplasia, ulceration with mixed inflammatory infiltrate consisting of macrophages, PMN's and eosinophils. In contrast the cecum from IL-7(-/-)RAG-2(-/-) mice revealed no lesions. Histologically both groups of mice showed H. hepaticus in direct contact with the epithelium. These data indicate that in the absence of lymphocytes IL-7 exacerbates an inflammatory response in the bowel after exposure to an infectious flora. IL-7 directly or indirectly activates myeloid cells or possibly NK cells and results in a pathological inflammation without obvious clearance of the suspected microbial organism.

Defective GM-CSF/IL3/IL5 receptor common β chain expression with or without AML associated with respiratory insufficiency.

Uta Dirksen, Uwe Hattenhorst, Horst Schroten, Alfred Böcking, Ryuichi Nishinakamura*, Richard Murray* and Stefan Burdach.
Children's Hospital Med. Center, Heinrich-Heine-Univ. Düsseldorf, Germany; * Dept. Immunol. DNAX Research Inst., Palo Alto CA 94304, USA.

Deficiency of the GM-CSF/IL3/IL5 receptors common β chain (βc) is a cause of fatal respiratory failure. βc deficiency can manifest as pulmonary alveolar proteinosis (PAP). PAP has heterogenous etiologies which may be genetic or acquired. Some cases of PAP have been reported to be associated with hematologic malignancies such as AML. In mice, we have generated the PAP phenotype by targeted deletion of the gene encoding the GM-CSF/IL3/IL5 receptors common β chain. In βc -/- mice steady state hematopoiesis was normal except for lower eosinophils. The fact that βc is almost exclusively expressed in myeloid cells provides evidence for a casual relationship between the lung disease and the hematopoietic system. Here, we describe an expression defect of βc or βc plus GM-CSF receptor α chain (GM-CSFR α) in three pediatric patients with AML and PAP-symptoms. All patients' leukemic cells failed to express normal levels of βc. Patients' 2 and 3 leukemic cells additionally lacked the expression of GM-CSFR α as shown by flow cytometry. Strinkingly reduced or absent function of βc was demonstrated in clonogenic progenitor assays with absent CFU growth following GM-CSF or IL3 stimulation. The response to growth factors acting via a growth factor receptor distinct from the GM-CSF/IL3/IL5 system (rhG-CSF) was normal. Following anti-leukemic treatment the pulmonary symptoms resolved and βc and GM-CSFR α expression was normal. Our findings provide evidence that a defect in the expression of βc or βc plus GM-CSFR α on AML blasts can be associated with pulmonary insufficiency in patients with AML.

Adventitial gene transfer of the antisense to senescent cell-derived inhibitor 1 results in increased neointima

S. Nikol, T.Y. Huehns, E. Krauß, S. Esin, S. Mrochen, M.G. Engelmann, M. Schmid, *W.H. Günzburg, B. Höfling
Cardiology, Ludwig Maximilians University, 81377 Munich, Germany; and *Institute of Virology, University of Veterinary Medicine, 1210 Vienna, Austria

Senescent cell-derived inhibitor 1 (*sdi*), also known as p21, prevents cells from entering the cell cycle and thereby blocks cell proliferation. The aim of this study was to prevent SDI synthesis by local delivery of an expression vector carrying the *antisense* sequence of *sdi* („*ids*") in a porcine restenosis model after injury. Porcine iliac artery segments were balloon-injured *in vivo* in a standard manner. Adventitial liposomal plasmid delivery was performed using the needle injection catheter (n=18). Balloon-injured segments served as controls (n=144). Segments were excised 7 days, 3 weeks or 4 months later.
Transfer and gene expression were demonstrated by (RT-) PCR. Transfection efficiency was determined to about 1:1000 cells by β-galactosidase staining. Tissue sections were examined at histology, with computer-assisted morphometry and immuno-histochemistry. After 21 days *ids*-treated vessels demonstrated a significant increase in neointimal hyperplasia (0.17±0.03mm^2 [SEM] area of neointima in balloon-injured controls segments versus 0.46±0.07mm^2, p<0.01, in *ids*-treated segments). The neointimal area within the external elastic lamina was 2.12±0.79% in controls versus 7.73±2.39% in *ids*-treated vessels (p<0.01). At the four-months time point, this difference was not apparent. Additionally, clusters of cells were stained using the proliferation marker Ki67 suggesting an enhanced proliferation of single transfected cells by *sdi*-antisense mRNA.
Liposome-mediated adventitial gene transfer leads to a transient effect on neoinitima formation. The success of gene delivery in this model underlines the potential for therapy with other genes.

RECRUITMENT OF TUMOR SPECIFIC CYTOTOXIC T CELLS (CTL) BY VACCINATION WITH GENETICALLY MODIFIED CELLS.

A. Lindemann[1], H. Veelken[1], A. Mackensen[1], M. Lahn[1], B. Franke[1], G. Koehler[2], P. Kulmburg[1], F.M. Rosenthal[1], E.H. Farthmann[3], R. Mertelsmann[1]. - 1) Dept. of Internal Medicine I (Hematology /Oncology), 2) Dept. of Pathology, 3) Dept. of General Surgery; Freiburg University Medical Center, D-79106 Freiburg, Germany.

Tumor cells transfected to express immunostimulatory cytokines or being admixed with similarly modified bystander cells are able to induce immune responses against unmodified tumor cells in animal models. In order to translate this approach into the clinic, a vaccine composed of autologous tumor cells and interleukin-2 (IL-2) gene transfected allogeneic fibroblasts (Fb) was evaluated in a phase I trial. 15 patients (pts.) received 3-4 injections of $2x10^6$ autologous tumor cells and a similar number of KMST6 Fb that stably secreted 5290 IU IL-2/10^6cells/24h.-Increa-sing transient inflammatory responses without systemic toxicity developed at vaccination sites and after injection of irradiated tumor cells only ($p<0.05$). These sites contained a dense infiltrate of $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cell lines and clones isolated from vaccination sites (VIL) of 3 out of 4 pts. (2 melanoma, 2 renal cell carcinoma) of whom permanent autologous tumor cell lines were available exhibited a dominant lytic activity against autologous tumor cells in vitro. In pt. 002 VIL contained a major population of CTL expressing the T cell receptor (TCR) β-chain Vβ21.3. This population, that was identified to be clonal by TCR-β-chain sequencing, was similarly found to infiltrate a skin metas-tasis. Since these cells were undetectable at sites of vaccination and delayed type hypersensitivity testing at treatment start while being dominant and readily detectable after therapy, it is likely that vaccination with autologous tumor cells plus IL-2 gene transfected allogeneic Fb had not only induced local accumulation but also an increase of the fequency of circulating tumor specific CTL.

GENETICALLY MODIFIED EBV-SPECIFIC CYTOTOXIC T CELLS FOR ADOPTIVE TRANSFER TO PATIENTS WITH EBV-POSITIVE HODGKIN DISEASE. Roskrow MA, Suzuki N, Heslop HE, Hudson M, Brenner MK, Rooney CM, St Jude Children's Research Hospital, Memphis, TN.

We have shown that adoptive transfer of EBV-specific cytotoxic T lymphocytes (CTLs) is effective as a treatment of EBV-positive immunoblastic lymphomas in immunocompromised hosts. As these tumors express all 9 of the EBV latency proteins, they are highly immunogenic and survive only in patients with a compromised immune system. Other EBV-related neoplasms such as Hodgkin disease express only the weakly immunogenic proteins EBNA1, LMP1 and LMP2a. As a result, these tumors can survive in a host with relatively normal immune function.To test the hypothesis that EBV-specific CTL lines can be generated Hodgkin patients and will kill Hodgkin tumor cells in vivo, we have generated 13 autologous EBV-specific CTL lines for treatment of patients with EBV-positive Hodgkin disease. Immunophenotypic analysis showed that the lines consisted of 93-100% CD3, 5-7% CD4 and 80-89% CD8-positive cells. Cytotoxicity assays showed 68-79% specific

killing of autologous lymphoblastoid cell lines and only 5-7% killing of HLA mismatched targets. We have treated 2 relapsed Hodgkin patients with autologous EBV-specific CTLs. To track the persistence of the infused CTLs, they were genetically marked with a retrovirus vector containing the neomycin gene. Semi-quantitative PCR analysis demonstrated that the marker gene was present in the circulating mononuclear cells for 12 weeks post infusion. In both patients, the level of EBV DNA fell >200 fold and the EBV-specific CTL precursor frequency increased 10 fold. Both patients had resolution of type B symptoms and temporary stabilization of pulmonary disease.

Development of gene transfer strategies for the treatment of pediatric malignancies.

Thomas Felzmann, Maria Buchberger, Ralph Kircheis, Ernst Wagner, Helmut Gadner. CCRI, St.Anna Kinderspital, Vienna, Austria, A-1090
Boehringer Ingelheim R&D, Vienna, Austria, A-1120

We compared the efficiencies of two gene transfer systems for the ex vivo transduction of primary cells. Both gene transfer systems employ the well established concept of transferrin-dependent receptor-mediated endocytosis. Polylysine (pL) or polyethylene imine (PEI) molecules are linked to transferrin to complex plasmid-DNA carrying a luciferase reporter gene. We found high transduction efficiencies for human and murine neuroblastoma cell lines (10^6 -10^7 relative light units). In contrast, primary human neuroblastoma cells in short term culture showed low and highly variable transduction efficiencies (10^2 -10^5 relative light units). We also studied the transduction efficiency of primary human fibroblasts and keratinocytes as a potential substitute for transgenic tumor cells as the source of cytokines. Transduction efficiencies were in the same range as the transduction efficiencies of murine and human tumor cell lines (10^6 -10^7 relative light units). Comparing the various PEI based complexes we observed best results using PEI coupled to two molecules of transferrin which was approximately equivalent to the pL based complexes; PEI alone was inefficient.

CD40 Ligand as a Co-Stimulator Molecule for the Generation of an Antitumor Response Dilloo D[1,2], Brown M[2], Zhong W.[2], Brenner M.[2] Heinrich-Heine University Düsseldorf, St. Jude Children's Research Hospital,Memphis

Leukemic cells may escape immune surveillance by their lack of co-stimulatory molecules. CD40 ligand (gp39) is a cell surface molecule expressed by T cells and antigen presenting cells that acts as a co-stimulator molecule for T and B cell responses. This activity led us to investigate whether transgenic expression of the CD40 ligand (CD40L) can induce systemic anti-leukemic activity against the otherwise non-immunogenic A20 B cell leukemic cell line. We found that in the presence of transgenic CD40L, A20 leukemia cells upregulate expression of B7.1 (CD28), Class I and II MHC, effects that favor successful tumor-antigen presentation. There was also upregulation of FAS and enhanced apoptosis allowig for uptake and presentation of tumor-antigens by professional antigen-presenting cells. We then used an A20 tumor immunisation model to show that expression of CD40L at one tumor site significantly delays growth of pre-existing tumor ($p<0.05$) in comparison to the control. Because CD40L also upregulates T cell function directly, we analyzed the effects on tumor growth and survival after combining CD40L with the T cell growh factor IL2. In the same model, the response to pre-existing distal tumor was significantly greater in mice treated with a combination of these co-stimulatory molecules (CD40L+IL2) than with either agent alone ($p<0.005$ in comparison to IL2 and CD40L alone). The CD40L and IL2-mediated anti-tumor effect was dependent on intact CD4+ and CD8+ T lymphocyte and NK cell systems, as depletion of either of these lymphoid subsets by antibody injection partially abrogated the anti-leukemia response. The enhanced response occured even though the cytokine and the co-stimulatory surface molecules were expressed on accessory cells separate from the tumor cells, simplifying the logistic demands of future clinical protocols that may adopt this dual-agent strategy.

Transgenic Expression of IL-2 in Peripheral Neuroectodermal Tumor Cells Yields Wild Type Tumor Cell Lysis

Barbara C. Engel, Hans-Jürgen Laws, Dagmar Dilloo and Stefan Burdach
Laboratory for Experimental Hematology and Stem Cell Transplantation; Department of Pediatric Hematology/Oncology; Heinrich Heine University Medical Centre, Düsseldorf

Systemic Interleukin-2 (IL-2) therapy in peripheral neuroectodermal tumor patients is often associated with severe side effects, in particular leukopenia and infection. Gene targeting experiments suggest that IL-2-induced immunodeficiency may be due to polyclonal T cell activation and induction of apoptosis. An alternative to systemic IL-2 therapy would be to achieve a local cytokine production in the proximity of the tumor, thereby expanding tumor restricted effector cells that can recirculate and reach distant sites of metastasis. In ^{51}Cr cytotoxicity assays we were able to demonstrate Ewing Tumor cell lysis by allogenous as well as autologous mononuclear cells (MNCs) previously stimulated by exogenous addition of IL-2. We transfected Ewing tumor cells as well as fibroblasts with an IL-2 gene expression vector using a cationic liposome reagent. Upon using IL-2 secreting fibroblasts as source of IL-2 for priming MNCs we obtained tumor cell lysis in the ^{51}Cr cytotoxicity assays . Stimulating MNCs in coculture with IL-2 secreting peripheral neuroectodermal tumor cells yielded wild type tumor cell lysis at a comparable level to that achieved by priming MNCs with exogenously adding 30 IU/ml IL-2. In patients receiving systemic IL-2 therapy, the average level of IL-2 measured in serum during application of the highest dose was 20 IU/ml. Our findings provide evidence that transgenic expression of IL-2 in peripheral neuroectodermal tumor cells yields wild type tumor cell lysis. Thus, transgenic expression of IL-2 within the tumors may expand tumor infiltrating effector cells without polyclonal T cell activation and apoptosis induction

Retroviral transfection of the human TNF-α cDNA into glioblastoma cell lines: MHC-upregulation and TNF-resistance

HB. Prisack, V. Balz, K. Martin, D. Lennartz, F. Weber, H. Bojar; Oncology Heinrich Heine University, Universitätsstraße 1 40001 Düsseldorf (Germany)

TNF-α directly stimulates immune effector cells and potentiates the expression of HLA-I, HLA-II and ICAM-1 on human glioblastoma cell lines. One aspect of immunotherapeutic strategies is to strengthen the tumor-effector cell affinity by upregulation of the

MHC related surface antigens and to amplify the presentation of tumorspecific antigens. In vitro experiments with exogenous cytokines like IFN-γ or TNF-α displayed a cytokin related expression of the HLA complex molecules. METHODS: To overcome the toxic effects and the short half-live of cytokines in vivo, we have cloned the cDNA of TNF-α in a MoMuLV derived vector, transfected five human glioblastoma lines and screened them for stable TNF-a producing cells. Cells were cultured for one month and then analysed for expression of HLA-I, HLA-II, ICAM-1 and CD44. RESULTS: Upregulation of HLA and ICAM-I by TNF-a cytokin expressing tumor cells was below the effect of exogenous recombinant TNF-α exposure. Incubation of TNF transfected tumor cells with exogenous TNF could not restore the original TNF sensitivity of ICAM-1 regulation. We propose that cells go through a TNF adaptation and that TNF-receptor mRNA is still transcribed in the glioblastoma, but the protein does not reach the cell surface. The results show that long-term culture of TNF transfected tumor cells deminished the TNF effects on intracellular molecules.

RELEASE OF IL-10 AND EXPRESSION OF ITS RECEPTOR IN CELLS OF THE EWING´S SARCOMA GROUP OF TUMOURS

F.van Valen, V.Kentrup, B.Truckenbrod, M.Schneider*
A.Borowski*, W.Winkelmann, H.Jürgens**

Laboratory of Experimental Orthopaedic Research and **Department of Paediatric Oncology and Haematology, Westfälische Wilhelms-University, Münster; *Laboratory of Immunology, Heinrich-Heine-University, Düsseldorf

Cytokines represent important agents in the treatment of neoplasia either when applied systemically, when locally injected at the tumor site, or when produced by tumor cells themselves upon gene transfer. Before transferring cytokine genes into tumor cells for the benefit of cancer therapy, however, it is crucial to analyse which cytokines are constitutively released and which cellular and molecular mechanisms are involved in such an expression by tumor cells. IL-10 is an immunoregulatory cytokine mainly produced by T-cells and monocytes. Herein we report that Ewing tumour (ET) cells (Ewing´s sarcoma; PNET) produce IL-10 in vitro. Production of the cytokine was up-regulated following phorbol 12-myristate 13-acetate (PMA) treatment and to a lesser extent upon γ-irradiation of cells. Calphostin C, a protein kinase C inhibitor, prevented PMA action but not irradiation-stimulated IL-10 release. Priming of cells with interferon (IFN)γ potently reduced both PMA and radiation-mediated IL-10 formation. Herbimycin A (a protein tyrosine kinase inhibitor) but not AACOCF3 (a specific phospholipase A2 inhibitor) and MK-866 (a specific lipoxygenase inhibitor) inhibited the production of IL-10. The antioxidant and NFκB inhibitor pyrrolidine dithiocarbamate (PDTC) prevented IL-10 release induced by PMA and radiation. FACS analysis revealed the expression of IL-10 receptors on ET cells. However, IL-10 receptors do not seem to play a role in either ET cellproliferation or cytokine (IL-6, TNFα) production. Collectively, IL-10 production in ET cells involves PKC-dependent and PKC-independent pathways that converge in a reactive oxygen species-mediated pathway. Our data provide a challenging model for investigating IFNγ as an anti-immunosuppressive agent in ET cell function.

Role of Apoptosis in CD3xCD19 Bispecific Antibody Induced T Cell Cytotoxicity against ALL Cells

C.F. Classen*, G. Moldenhauer**, and K.-M. Debatin*
*Hematology/Oncology, University Children's Hospital, Division of Mol. Oncology and **Division of Mol. Immunology, German Cancer Research Center, Heidelberg, Germany

Bispecific antibodies containing CD3 and CD19 binding sites within a single antibody molecule (CD3xCD19) can direct T cells via the T cell receptor complex to CD19 expressing malignant B cells. CD3xCD19 were used to activate peripheral blood mononuclear leukocytes (PBMC) from healthy donors. Activated PBMC were used as effectors on different CD19-positive human lymphoma cells (Nalm6, Reh). In a flow-cytometry-based assay (with annexin V and propidium iodide) significant specific cytotoxicity in targets could be shown. The caspase (ICE-protease) inhibitor Z-Val-Ala-Asp-CH_2F (zVAD) inhibited kill of Nalm6 cells in a dose-dependant manner. Since annexin V is an indicator of apoptosis-related membrane-breakdown and caspases are key enzymes in the apoptosis inducing signalling cascade, these results suggest a role of apoptosis pathways in bispecific antibody-mediated cytotoxicity. In the human T leukemia cell line CEM, it has been shown that doxorubicin-induced apoptosis involves the CD95 system and, thus, CD95 resistant CEM cells (CEMapoR) are also resistant to doxorubicin. Now comparing LAK killing of CEMapoR and doxorubicin resistant CEM (CEMdoxoR) to killing of the parental sensitive CEM cells (CEMS), both resistant cells were significantly less susceptible. In the presence of zVAD, CEMS cells became significantly more resistant, while CEMapoR and CEMdoxoR cells showed no change.
Our data demonstrate that CD3xCD19 can be used to induce T cell cytotoxicity against leukemic blasts. Since T cell kill depends on residual intact apoptosis pathways in the targets, cells resistant to cytostatic drugs might be equally resistant to immunological approaches.

VIRAL PARTICLES WITH HETEROLOGOUS BINDING MOTIFS: AN APPROACH TO SPECIFICALLY ALTER THE TROPISM OF THE B-LYMPHOTROPIC PAPOVAVIRUS

Josmar Langner and Michael Pawlita
Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany; Fax: (49) 6221 424932; E-mail: J.Langner@dkfz-heidelberg.de

We attempt to construct a model system for a cell and tissue specific viral vector with predefined specificity. The host cell tropism is to be specifically altered by grafting a heterologous receptor binding motif onto the viral capsid. The B-lymphotropic papovavirus (LPV) with its very narrow, receptor-regulated host range, that is limited to B-lymphoma-derived cell-lines, is a good starting point for such an approach. LPV's major capsid protein VP1 shares more than 55 % of primary sequence homology with murine polyomavirus (PyA2) and simian virus 40 (SV40). The x-ray structures of PyA2 in complex with a
receptor fragment and that of SV40 but not of LPV have been determined. The putative structure of LPV-VP1 has been generated by homolgy modelling, using the x-ray derived coordinates of PyA2 and SV40. Arginin-glycin-aspartic acid (RGD) sequences are to be cloned on top of the predicted solvent-exposed loops. Such RGD triplets are common motifs in cell-cell and cell-matrix-interactions. They mediate the binding of a wide range of proteins, especially, but not only to (V-(3-type integrins and are also ligands of coxsackie-virus serotype A9 (CAV-9) and foot and mouth disease virus (FMDV), necessary for virus attachment and probably sufficient for uptake and cell entry. The mutant LPV-VP1 is to be expressed in a baculovirus vector as well as in an early replacement LPV vector. In this LPV-CAT construct the viral T-antigen ORF is replaced by the CAT reporter gene, whereas T-antigen is provided in trans by an inducible helper cell-line. The mutant proteins are to be checked for correct folding, particle assembly, exposition of the RGD-motif, for altered binding affinitiy towards (V-(3-type integrin presenting cells and finally for infection
of such cells.

A novel and universal applicable technique for quantitative PCR

CN. Wang, K. Wu., M. Traub* and H.-T. Wang
Biotronics Corporation, Lowell Massachusetts
*Department of Endocrinology and Developmental Physiology, Heinrich-Heine-University of Düsseldorf

This technique can be used as a real time PCR quantitation tool. Based on a fluorescence energy transfer mechanism, this method monitors changes in duplex formation between complementary nucleic acid strands. The two complementary strands are labeled with donor and acceptor fluorophores. Fluorescence energy transfer is facilitated when the strands are base-paired, or eliminated when the double stranded formation is dissociated. Within the PCR reaction, the change of fluorescence can be correlated to the amount of initial target dosage by using a defined standard. The fluorescence changes can be monitored directly and cycle by cycle. The assay itself is following a simple two step protocol. In the first step asymmetric PCR is carried out to the late log Phase before one of the target strands is significantly overproduced. In the second step a labeled duplex primer, complementary to the overproduced target strand, is added. As the semi-nested amplification proceeds, the labeled duplex starts to dissociate. The measured fluorescence intensity is in proportion to the amount of the labeled duplex left at the end of each amplification cycle. The decrease in the fluorescence intensity correlates to the initial target dosage and the extent of amplification. This novel method is applicable for genetic diseases and gene dosage quantitation.

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